

fails to select peptides w/ back response

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(54) Title: DIAGNOSTIC METHOD

(57) Abstract: Method of diagnosing in an individual recent exposure to an agent which is a pathogen, vaccine or any other moiety which induces a cellular response, said method comprising determining in vitro whether the T cells of the individual recognise a protein from said agent having a length of at least 30 amino acids, to a greater extent than one or more peptide epitopes from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

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## DIAGNOSTIC METHOD

### Field of the invention

The invention relates to a method of diagnosing the infection status of individuals.

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### Background of the invention

Infection by a pathogen may or may not cause disease symptoms in an individual. Although therapeutic products are available for treating infection by pathogens, prolonged use of these products may be harmful. Therefore it is desirable to target use of the therapeutic products to individuals who are more likely to develop disease symptoms. Targeting therapy in this way will also be more cost-effective.

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### Summary of the invention

Long term infected individuals who have not developed disease symptoms are much less likely to develop disease symptoms than recently exposed individuals. For example following exposure to M. tuberculosis individuals have a risk of approximately 10% of progressing to active tuberculosis with disease symptoms in the first one to two years following exposure. If active tuberculosis does not manifest within the first one to two years then the residual risk of progress to active tuberculosis is 5% over the remaining lifetime of the individual. It is therefore desirable to target recently infected individuals for preventative treatment because they have a high probability of progressing to disease.

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Some groups of individuals have a higher risk for developing active tuberculosis, such as young children (less than 5 years old), newborn babies (less than 1 year old), individuals with HIV infection or on immunosuppressive medication such as corticosteroids (typically oral corticosteroids), such as prednisolone, or antibodies against TNF- $\alpha$  (typically monoclonal and/or humanised), such as infliximab. It is even more important to diagnose recent exposure to pathogen in such individuals.

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The inventors have found that T cells from individuals recently exposed to an intracellular pathogen react to whole proteins from the pathogen but do not react to, or show substantially less reaction to, peptide epitopes from the pathogen. It is believed that this may be because when a cellular T cell immune response has just been primed

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(induced) by a recent infection the T cells are of a lower affinity for their cognate ligand, as fine-tuning of the epitope specificity and clonal expansion of the different T cell populations is still going on. Since in T cell response assays whole protein is presented to T cells after uptake and processing by antigen presenting cells (APCs) followed by presentation of the optimal peptide epitopes in the context of MHC molecules at the APC surface, even cells of relatively low affinity will recognise these optimum naturally processed and presented epitopes.

In contrast short multiple peptide epitopes from the pathogen even when they together represent the entire sequence of a protein antigen are not normally the optimal epitopes, but merely tend to contain the optimum epitope sequence within their sequence. Therefore recognition of these peptides will require T cells to be present which are of higher affinity to the optimal epitope. It is believed that such T cells only appear in the later course of infection, when the T cell repertoire is more mature and focussed, and would not be present in recently exposed individuals.

Accordingly the invention provides a method of diagnosing in an individual recent exposure to an agent which is a pathogen, a vaccine or a moiety which causes a cellular immune response, said method comprising determining in vitro or in vivo whether the T cells of the individual recognise a protein from said agent having a length of at least 30 amino acids to a greater extent than a peptide epitope from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

Preferably in the method the pathogen is *M. tuberculosis* and the peptide epitope and/or protein is from ESAT-6 or CFP10.

The inventors have also shown using a T cell detection (ex vivo ELISPOT) based technique that T cells from the individuals exposed to a pathogen reacted to antigen from the pathogen at 3 months from exposure, but no longer reacted to antigen at 6 months from exposure. Given that the presence of effector T cells indicates the presence of infection by a pathogen this shows these individuals had cleared the infection which had been detected initially. This elucidation of the dynamics of the T cell response during infection and clearance of infection shows the need to test at a subsequent time point to avoid treating individuals who naturally clear infection.

Accordingly the invention provides a method of diagnosing an individual who

has cleared an infection by a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the individual has cleared the infection.

In addition the inventors have identified individuals whose T cells did not react at 3 months from exposure but did react at 6 months from exposure. These individuals are mounting a slower weaker response to infection. They are therefore less likely to be able to control the infection and are more likely to progress to active disease. It is therefore desirable to target this group of individuals for treatment.

Accordingly the invention provides a method of diagnosing an individual who is more likely to progress to active disease after exposure to a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and subsequent second time point after exposure to the pathogen, wherein the finding that the T cells do not recognise the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is more likely to progress to active disease.

The finding may also be used in a method of diagnosing an individual who mounts a weaker response to a vaccine or a moiety which induces a cellular response after exposure to the vaccine or moiety comprising determining whether the T cells of the individual recognise antigen from the vaccine or moiety at a first and subsequent second time point after exposure, wherein the finding that the T cells do not recognise the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is mounting a weaker response to the vaccine or moiety.

The inventors have also shown advantages in detection of latent mycobacterial infection by using the T cell detection assays described herein in individuals on immunosuppressive therapy or about to start immunosuppressive therapy.

Accordingly the invention provides a method of diagnosing susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on or about to start immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis

disease and latent mycobacterial infection.

In addition the invention provides a method of monitoring susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis disease and latent mycobacterial infection.

The inventors have shown that effector T cells specific for mycobacterial antigen increase markedly prior to the onset of active symptomatic mycobacterial infection. Thus detection of this increase in effector T cells may be used as a predictor of progression, or susceptibility to progression to disease in asymptomatic latently infected individuals.

Accordingly the invention provides a method of detecting susceptibility to onset of active mycobacterial disease in an individual who does not have any symptoms of mycobacterial disease comprising determining whether the individual has increased levels of T cells which recognise a mycobacterial antigen, to thereby determine whether the individual is susceptible to onset of active mycobacterial disease. This would allow doctors to initiate radiological and other investigations in asymptomatic individuals with latent mycobacterial infection, and to diagnose and treat early active disease before the onset of symptoms. Such early treatment, even before symptoms have begun, would prevent considerable morbidity and mortality, especially for (but not limited to) people with multi-drug resistant tuberculosis.

#### **Detailed description of the invention**

Different aspects of the invention are discussed below. It is to be understood that the specific T cell detection techniques which are discussed below in the context of one aspect of the invention (particularly the method of diagnosing recent exposure) may be used in other aspects of the invention. Thus the description of the types and numbers of peptide epitopes/proteins (including their length, origin, sequence and analogues) used in the detection method, the manner in which the reaction of the T cell is detected (including change of state), the types of T cell samples which are used, the way such samples may be processed, the form of the T cells in the assay and the

specific manner in which the T cell detection is carried out, that is given for the method of diagnosing recent exposure is also relevant to the other aspects of the invention.

Similarly, the types of individuals to be tested and the types of mycobacterium with which individuals may be infected discussed in the context of the method of  
5 diagnosing recent exposure is applicable to the other aspects of the invention.

*Method of diagnosing recent exposure*

The method of the invention comprises determining whether the T cells of an individual recognise a protein (with a length of at least 30 amino acids) from an agent  
10 to a greater extent than a peptide epitope from the agent. This may be done by detecting the reaction of the T cells either to the protein and peptide epitope or to an analogue of either of these (discussed below). It is understood that herein reference to "protein" or "peptide epitope" from the pathogen will also include the analogues of these molecules unless the context requires otherwise.

15 The method is generally performed on a sample from an individual who is preferably a human, but may be an animal (typically an animal which can be naturally or artificially infected by the relevant pathogen). Thus the individual may be a mammal, such as a primate, cow, sheep, pig, badger or rodent, e.g. a mouse or rat. The individual may be at risk of (natural) exposure to the pathogen, for example the  
20 individual may live in an area in which the pathogen occurs. The individual may have an increased risk of becoming infected, typically for socio-economic reasons or may have a genetic or acquired predisposition to the pathogen. In one embodiment the exposure is not a natural exposure (i.e. it is an artificial exposure), for example intentional exposure of an animal model to a pathogen. In another embodiment the  
25 exposure is to a non-natural (typically intentional) release of the pathogen in the area where the host (including humans) lives.

The pathogen may be an extracellular pathogen, but is preferably an intracellular pathogen, and is generally a naturally occurring pathogen (not modified artificially). The pathogen is typically able to infect any of the specific species of host  
30 mentioned herein. It may be a virus, bacterium or fungus, such as HPV, HIV, HCV, a Chlamydia species, HBV, EBV, CMV, VZV, HSV, Legionella, S. typhi, P. falciparum, Leishmaniasis, M. leprae, influenza virus, foot and mouth virus, a Toxoplasma species,

a *Brucella* species, a *Cryptococcus* species, a *Candida* species or an *Aspergillus* species.

In a preferred embodiment the pathogen is a mycobacterium. The mycobacterium typically expresses ESAT-6 or CFP10, and may be *M.tuberculosis*.

- 5 The mycobacterium may be *M.marinum* or *M.kansasii*. The pattern of clinical symptoms can be used to distinguish between these two organisms and *M.tuberculosis*. The mycobacterium may be *M. bovis* (which infects cows, but can also infect humans and other species such as badgers and monkeys).

- 10 In the case where the agent is a vaccine the vaccine may contain antigen from (provide protection against) any of the pathogens mentioned herein. Any of the types of agent mentioned herein may be capable of inducing a cellular response in the individual, typically a T cell and/or NK cell response.

- The protein and/or peptide epitope of the pathogen may be of any of the pathogens mentioned herein, preferably being mycobacterial. In one embodiment the protein is, or contains sequence from, a particular pathogen protein, whilst the peptide  
15 epitope is from a different protein from the pathogen. However preferably the peptide epitope is an epitope within the protein, i.e. the protein comprises the sequence of the peptide epitope. The peptide epitope generally contains within its sequence an optimal epitope, typically flanked by one or more amino acids at the N or C terminal end of the  
20 optimal epitope sequence.

- The protein may be a membrane protein, a cytoplasmic protein (present in the cytoplasm of the pathogen or a cell which it has infected), a secreted protein (secreted from the pathogen and/or from the infected cell), an enzyme, a structural protein or a regulatory protein. The protein may be one which typically comprises at least 10%,  
25 such as at least 30% or 50% of the dry mass of the agent. The protein may be one which in its natural form and/or as used in the method comprises at least 5, such as at least 10 or 15 CD4 and/or CD8 T cell epitopes.

- The peptide epitope is typically a fragment of any of the pathogen proteins mentioned. In the case of *M. tuberculosis* the peptide epitope may be any of the  
30 peptides shown below from ESAT-6 and CFP-10.

Peptides from ESAT-6:

MTEQQWNFAGIEAAA  
WNFAGIEAAASAIQG  
IEAAASAIQGNVTSI  
5 SAIQGNVTSIHSLLD  
NVTSIHSLLDDEGKQS  
HSLLDDEGKQSLTKLA  
EGKQSLTKLAAAWGG  
LTKLAAAWGGSGSEA  
10 AAWGGSGSEAYQGVQ  
SGSEAYQGVQQKWD  
YQGVQQKWDATATEL  
QKWDATATELNNALQ  
TATELNNALQNLART  
15 NNALQNLARTISEAG  
NLARTISEAGQAMAS  
ISEAGQAMASTE GNV  
QAMASTE GNV TGMFA

20 Peptides from CFP-10:

MAEMKTDAATLAQEA  
TDAATLAQEAGNFER  
LAQEAGNFERISGDL  
25 GNFERISGDLKTQID  
ISGDLKTQIDQVEST  
KTQIDQVESTAGSLQ  
QVESTAGSLQGQWRG  
AGSLQGQWRGAAGTA  
30 GQWRGAAGTAAQAAV  
AAGTAAQAAVVRFQE  
AQAAVVRFQEAANKQ



VRFQEAANKQKQELD  
 AANKQKQELDEISTN  
 KQELDEISTNIRQAG  
 EISTNIRQAGVQYSR  
 5 IRQAGVQYSRADEEQ  
 VQYSRADEEQQQAALS  
 ADEEQQAALSSQMGF

The peptide epitope typically has a length of at least 8 to 29 amino acids, such  
 10 as 12 to 25 amino acids. The protein typically has a length of at least 30 to 400 amino  
 acids, such as 50 to 300, or 80 to 200 amino acids in length. The protein may be the  
 same as the whole naturally occurring protein, or a fragment thereof. In one  
 embodiment it is in the form of a fusion protein, for example with non-pathogen protein  
 sequence. Generally the protein comprises a pathogen sequence (sequence from a  
 15 protein of the pathogen) which is at least 8, for example at least 12, 18, 25 or 30 amino  
 acids long.

The method of the invention may be performed using any suitable technique.  
 Different techniques are discussed below and include techniques which detect the  
 reaction of T cells or which quantitate antigen specific T cells. These techniques may  
 20 be based on detection of 'spots' of a substance secreted from T cells (such as  
 ELISPOT), sorting (counting) of T cells (for example using intracellular staining or  
 FACS), use of MHC tetramers (for example in a sorting technique) or an ELISA  
 technique.

The method of the invention is generally based on the detection of different  
 25 levels of response from and/or different frequencies of T cells in an individual to one or  
 more proteins and one or more (smaller) peptide epitopes from a pathogen. The T cells  
 which react are specific for/bind to amino acid sequence in the protein or peptide  
 epitope. The T cells which are analysed in the method may be CD4 and/or CD8 T  
 cells,  $\gamma\delta$  T cells or CD1 restricted T cells. The T cells have been pre-sensitised *in vivo*  
 30 to protein from the pathogen.

The method of the invention may be performed using a technique which detects  
 T cell reaction to a protein/peptide epitope. In many such techniques whether or not

the T cells of the individual react to the protein or peptide epitope will be readily apparent, and thus individuals will be diagnosed as having been recently exposed if their T cells react to the protein and do not react to the peptide epitope. Suitable thresholds may be determined by the skilled persons. In one embodiment arbitrary thresholds are used to determine positive and negative responses.

Typically the method will be performed in a manner in which reactive T cells present at a frequency of at least about 20 per million peripheral blood mononuclear cells (PBMCs) will be detectable (a positive result), and preferably distinguishable from a reactive T cells present at a frequency of about 19 per million PBMCs or less (a negative result).

Thus individuals will typically be selected as being exposed recently to pathogen if they are found to have T cells which are able to recognise the protein at a frequency of at least 20 per million PBMCs and if they are found to have less than 19 per million PBMCs which recognise the peptide epitope. It is understood though that a positive and negative result may be defined using thresholds different from these specific thresholds.

In a preferred embodiment the T cells are detected by:

(i) contacting in vitro or in vivo a first population of T cells from the individual with one or more peptide epitopes from the pathogen (including an analogue of said peptide which is recognised by T cells that recognise said peptide), and determining the reaction of the T cells to the peptide epitope(s), and

(ii) contacting in vitro or in vivo a second population of T cells from the individual with a protein from the pathogen (including an analogue of said protein which is recognised by T cells that recognise said protein), wherein the protein has a length of at least 30 amino acids and determining the reaction of the T cells to the protein.

Determination of whether the T cells react to/recognise the protein or peptide epitope is may be done by detecting a change in the state of the T cells in the presence of the protein or peptide epitope. The change in state is generally caused by antigen specific functional activity of the T cell after the T cell receptor binds the protein (after it is processed) or peptide epitope. Generally when binding the T cell receptor the processed protein or peptide is bound to an MHC class I or II molecule, which is

typically present on the surface of an antigen presenting cell (APC).

The change in state of the T cell may be the start of or increase in secretion of a substance from the T cell, such as a cytokine, especially IFN- $\gamma$ , IL-2 or TNF- $\alpha$ .

Determination of IFN- $\gamma$  secretion is particularly preferred. In one embodiment more than one cytokine is detected, such as 2, 3, 4 to 10 or more cytokines. Intracellular changes may be detected, for example by using intracellular staining techniques, typically intracellular cytokine staining (e.g. for any of the cytokines mentioned herein). The staining can be detected using a cell sorting technique, for example using a FACS technique.

10 The substance can typically be detected by allowing it to bind to a specific binding agent and then measuring the presence of the specific binding agent/substance complex. The specific binding agent is typically an antibody, such as polyclonal or monoclonal antibodies. Antibodies to cytokines are commercially available, or can be made using standard techniques.

15 Typically the specific binding agent is immobilised on a solid support. The support may be a well (typically in an assay plate) or may be a microsphere. In one embodiment this allows the actual number of responding T cells to be determined since after binding the agent the substance will remain in the vicinity of the T cell which secreted it. Thus 'spots' of substance/agent complex may form on the support, each  
20 spot representing a T cell which is secreting the substance. Quantifying the spots (and typically comparing against a control) allows determination of recognition of the peptide.

After the substance is allowed to bind, the solid support can optionally be washed to remove material which is not specifically bound to the agent. The  
25 agent/substance complex may be detected by using a second binding agent which will bind the complex. Typically the second agent binds the substance at a site which is different from the site which binds the first agent. The second agent is preferably an antibody and is labelled directly or indirectly by a detectable label.

Thus the second agent may be detected by a third agent which is typically  
30 labelled directly or indirectly by a detectable label. For example the second agent may comprise a biotin moiety, allowing detection by a third agent which comprises a streptavidin moiety and typically alkaline phosphatase as a detectable label.

In one embodiment the detection system which is used is the *ex-vivo* ELISPOT assay described in WO 98/23960. In that assay IFN- $\gamma$  secreted from the T cell is bound by a first IFN- $\gamma$  specific antibody which is immobilised on a solid support. The bound IFN- $\gamma$  is then detected using a second IFN- $\gamma$  specific antibody which is labelled with a detectable label. Other detectable labels may be used.

In another embodiment detection is performed using a multiplex analysis of cytokines performed using microspheres coated with antibody specific to a cytokine. Detection antibodies (that bind to the cytokine bound to the antibody on the microsphere) are may be used. Such detection antibodies may be labelled, for example with a fluorescent label. The detection technique may be based on the Luminex multiplex cytokine detection system.

Typically the T cells used in the method are taken from the individual in a blood sample, although other types of body sample which contain T cells can be used. The sample may be added directly to the assay or may be processed first. Typically the processing may comprise diluting of the sample, for example with water or buffer. Typically the sample is diluted from 1.5 to 100 fold, for example 2 to 50 or 5 to 10 fold.

The processing may comprise separation of components of the sample. Typically mononuclear cells (MCs) are separated from the samples. The MCs will comprise the T cells and APCs. Thus in the method the APCs present in the separated MCs can present peptide to the T cells. In another embodiment only T cells, such as only CD4 T cells, can be purified from the sample. PBMCs, MCs and T cells can be separated from the sample using techniques known in the art, such as those described in Lalvani *et al* (1997) *J.Exp. Med.* 186, p859-865.

In the case of a blood sample, red blood cells may be removed from the sample (to leave serum and other cells).

In one embodiment the T cells which are detected are in the form of unprocessed or diluted samples. The T cells are preferably directly *ex vivo*, i.e. they are not cultured before being used in the method. The T cells are typically freshly isolated T cells (such as in the form of freshly isolated MCs or PBMCs).

The APC which is typically present in the method may be from the same individual as the T cell or from a different individual. The APC may be a naturally

occurring APC or an artificial APC. The APC is a cell which is capable of presenting peptide to a T cell. It is typically a B cell, dendritic cell or macrophage. It is typically separated from the same sample as the T cell and is typically co-purified with the T cell. Thus the APC may be present in MCs or PBMCs. The APC is typically a freshly  
5 isolated *ex vivo* cell or a cultured cell. It may be in the form of a cell line, such as a short term or immortalised cell line. The APC may express empty MHC class I or II molecules on its surface.

In one embodiment of the method more than one protein from the pathogen (typically at least 2, 5, 10 or more different proteins) and/or more than one peptide  
10 epitope from the pathogen (typically at least 2, 5, 10 or more different peptide epitopes) may be used. Thus, for example, the T cells can be placed into an assay with all the proteins or peptide epitopes (i.e. a pool of the proteins or peptides) which it is intended to test. Alternatively the T cells can be divided and placed into separate assays each of which contain one or some of the proteins or peptides which it is intended to test.

15 In one embodiment the protein or peptide epitope is provided to the APC in the absence of the T cell. The APC is then provided to the T cell, typically after being allowed to present the processed protein or peptide epitope on its surface. Presented peptide may have been taken up inside the APC and presented, or simply be taken up onto the surface without entering inside the APC.

20 The duration for which the protein or peptide epitope is contacted with the T cells will vary depending on the method used for determining recognition. Typically  $10^4$  to  $10^7$ , preferably  $1 \times 10^5$  to  $5 \times 10^5$  PBMCs are added to each assay. The peptide is typically used in the assay at a concentration of from  $10^{-1}$  to  $10^3 \mu\text{g/ml}$ , preferably 0.5 to  $50 \mu\text{g/ml}$  or 1 to  $10 \mu\text{g/ml}$ .

25 Typically the length of time for which the T cells are incubated with the protein or peptide is from 4 to 72 hours, preferably 6 to 48, 8 to 24 or 10 to 16 hours. When using *ex vivo* PBMCs it has been found that  $0.3 \times 10^6$  PBMCs can be incubated in  $10 \mu\text{g/ml}$  of peptide for 12 hours at  $37^\circ\text{C}$ .

The method may be based on an ELISA method, such as the whole blood  
30 Quantiferon system (for example as available from Cellestis).

In one embodiment instead of the protein and/or peptide epitope analogues are used which are recognised by T cells which recognise the protein or peptide. Thus

such analogues may be identified by routine means and their ability to be recognised by the relevant T cells can be tested using any suitable technique mentioned herein. For the proteins such recognition will of course be after processing and presentation of the protein and/or analogue by an APC.

5           The analogue will generally have similar binding properties to the protein and/or peptide and thus typically binds to the same MHC molecule. The analogue may bind to antibodies specific for the protein or peptide, and thus may inhibit binding of the protein or peptide to such an antibody.

          The analogue is typically a protein or peptide. It may have homology with the  
10   equivalent original protein or peptide. A peptide which is homologous to another peptide is typically at least 70% homologous to the peptide, preferably at least 80 or 90% and more preferably at least 95%, 97% or 99% homologous thereto, for example over a region of at least 8, preferably at least 15, for instance at least 40, 60 or 100 or more contiguous amino acids. The analogue typically differs from the protein or  
15   peptide by 1, 2, less than 6, such as less than 12 mutations (each of which is a substitution (e.g. a conservative substitution), deletion or insertion) for example over any of the above-mentioned lengths of region mentioned for homology.

          Methods of measuring protein homology are well known in the art and it will be understood by those of skill in the art that in the present context, homology is  
20   calculated on the basis of amino acid identity (sometimes referred to as "hard homology"). For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* 12, p387-395).

          An analogue which is a protein or peptide typically has any of the amino acid  
25   lengths mentioned above for the protein or peptide discussed above and/or may be part of a fusion protein. Typically the amino acids in the analogue at the equivalent positions to amino acids in the original protein or peptide which contribute to binding the MHC molecule or are responsible for the recognition by the T cell receptor, are the same or are conservative changes.

30           Conservative substitutions are defined in the table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Typically the analogue of the protein or peptide comprises one or more modifications, which may be natural post-translation modifications or artificial modifications. The modification may provide a chemical moiety (typically by substitution of a hydrogen, e.g. of a C-H bond), such as an amino, acetyl, hydroxy or halogen (e.g. fluorine) group or carbohydrate group. Typically the modification is present on the N or C terminus.

The analogues may comprise one or more non-natural amino acids, for example amino acids with a side chain different from natural amino acids. Generally, the non-natural amino acid will have an N terminus and/or a C terminus. The non-natural amino acid may be an L- or D- amino acid. The analogues typically has a shape, size, flexibility or electronic configuration which is substantially similar to the original protein or peptide. It is typically a derivative of the original protein or peptide.

The analogue is typically designed by computational means and then synthesised using methods known in the art. Alternatively the analogue can be selected from a library of compounds. The library may be a combinatorial library or a display library, such as a phage display library. The library of compounds may be expressed in the display library in the form of being bound to a MHC class I or II molecule, such as the MHC molecule which the original peptide binds. Analogues are generally selected from the library based on their ability to mimic the binding characteristics of the original protein or peptide. Thus they may be selected based on ability to bind a T cell receptor or antibody which recognises the original protein or peptide.

In one embodiment the T cells are detected not based on their response to a substance but based on their ability to bind a specific binding agent. Typically the

agent is or comprises any of the proteins, peptide epitopes or analogues mentioned herein. The agent may be labelled (for example using any of the detectable labels mentioned herein). The specific binding agent may comprise an MHC molecule, and is preferably an MHC tetramer-peptide complex.

5           The peptide or analogue discussed herein can be made using standard synthetic chemistry techniques, such as by use of an automated synthesizer. They can be made from a longer polypeptide, e.g. a fusion protein, which polypeptide typically comprises the sequence of the peptide, and may be derived from the polypeptide by for example hydrolysing the polypeptide, such as using a protease; or by physically breaking the  
10 polypeptide. The protein may be expressed recombinantly.

In the case where the method is performed in vivo the protein, peptide epitope and/or analogue may be administered by any suitable means and at any suitable dose, for example in the form, by the route or at the dosage discussed for the therapeutic product below. Administration to the skin is preferred.

15           The invention also provides a method of treating an individual comprising administering to an individual diagnosed as having been recently exposed to a pathogen by the diagnosis method a product which prevent or treats the condition caused by the pathogen. Thus the invention provides use of the product in the manufacture of a medicament for the treatment of an individual who has been diagnosed as having been  
20 recently exposed to the pathogen by a method of the invention. Typically a non-toxic effective amount of the therapeutic agent is administered.

In the case M. tuberculosis the therapeutic agent may be rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicylic acid, kanamycin, capreomycin, ethionamide, cycloserine, thiacetazone or a fluoroquinolone (e.g.  
25 ciprofloxacin).

The product may be in the form of a pharmaceutical composition which comprises the agent and a pharmaceutically acceptable carrier or diluent. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. Typically the product is administered by parenteral, intravenous, intramuscular,  
30 subcutaneous, transdermal, intradermal, oral, intranasal, inhalation (into the lungs), intravaginal, or intrarectal administration.

The dose of the product may be determined according to various parameters,



especially according to the particular agent; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. A physician will be able to determine the required route of administration and dosage for any particular patient. A suitable dose may however be from 10 $\mu$ g to 10g, for example from 100  $\mu$ g to 1g of the product.

*Methods of diagnosis or testing of vaccines comprising testing at two time points*

The inventors have also shown using a T cell detection (ex vivo ELISPOT) based technique that T cells from the individuals exposed to a pathogen reacted to antigen from the pathogen at 3 months from exposure, but no longer reacted to antigen at 6 months from exposure. Given that the presence of effector T cells indicates the presence of infection by a pathogen this shows these individuals had cleared the infection which had been detected initially. This elucidation of the the dynamics of the T cell response during infection and clearance of infection shows the need to test at a subsequent time point to avoid treating individuals who naturally clear infection.

Accordingly the invention provides a method of diagnosing an individual who has cleared an infection by a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the individual has cleared the infection.

The infection may be cleared naturally by the immune response of the individual but could also be cleared pharmacologically by use of a product which treats the infection.

In addition the inventors have identified individuals whose T cells did not react at 3 months from exposure but did react at 6 months from exposure. These individuals are mounting a slower weaker response to infection. They are therefore less likely to be able to control the infection and are more likely to progress to active disease. It is therefore desirable to target this group of individuals for treatment.

Accordingly the invention provides a method of diagnosing an individual who is more likely to progress to active disease after exposure to a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen

at a first and subsequent second time point after exposure to the pathogen, wherein the finding that the T cells do not recognise the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is more likely to progress to active disease.

5       The finding may also be used in a method of diagnosing an individual who mounts a weaker response to a vaccine or a moiety which induces a cellular response after exposure to the vaccine or moiety comprising determining whether the T cells of the individual recognise antigen from the vaccine or moiety at a first and subsequent second time point after exposure, wherein the finding that the T cells do not recognise  
10 the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is mounting a weaker response to the vaccine or moiety.

Further testing at a subsequent time point may also be used to test the efficacy of a vaccine. Accordingly the invention provides a method of testing the efficacy of a vaccine which has been administered to an individual comprising determining whether  
15 the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the vaccine antigen has been cleared and is not persisting.

If the vaccine is found to have low efficacy (i.e. is being cleared) then the  
20 individual may need to receive an additional or augmented dose of the vaccine or be vaccinated with an alternative vaccine. Thus the same vaccine may be administered to the individual again optionally at increased dose. Alternatively a different vaccine may be administered (for example containing different antigens/proteins).

In the above methods the first time point and second time point are typically  
25 separated by about 2 to 16 weeks, such as about 4 to 12 weeks. The first time point may be about 8 to 16 weeks (preferably about 12 weeks) after exposure and/or the second time point may be about 18 to 48 weeks (preferably about 24 weeks) after exposure.

The individuals who are diagnosed may be any of the individuals who are  
30 mentioned herein, but are preferably human. The said determining of T cell recognition may be carried out using any suitable method, such as any suitable method disclosed herein. The antigen may be any antigen which is recognised by T cells (such

as any type of T cell mentioned herein) and thus may be any of the proteins or peptide epitopes mentioned herein. Analogues of the antigen may be used in the determination such as any of the types of, or specific, analogues mentioned herein

Typically the pathogen is an intracellular pathogen such as any such pathogen  
5 mentioned herein, for example HPV, HIV, SIV, HCV, a Chlamydia species, HBV, EBV, CMV, VZV, HSV, Legionella, *S. typhi*, *P. falciparum*, Leishmaniasis, *M. leprae*, influenza virus, foot and mouth virus, a Toxoplasma species, a Brucella species, a Cryptococcus species, a Candida species or an Aspergillus species. Preferably the pathogen is *M. tuberculosis*.

10 As mentioned above the recognition of the antigen or analogue may be determined using any suitable method, but is preferably determined by detecting secretion of a cytokine from the T cells, such as IFN- $\gamma$ .

The above method which involves determining T cell recognition at two time points may be used to diagnose individuals who have a high probability of progressing  
15 to active disease. The invention provides a method of treating an individual who has been diagnosed in this way comprising administering to the individual a product which prevent or treats the condition caused by the pathogen.

Preferably the pathogen is *M. tuberculosis* and/or the agent is rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicylic acid,  
20 kanamycin, capreomycin, ethionamide, cycloserine, thiacetazone or a fluoroquinolone, or an analogue of such an agent.

The inventors also identified individuals who only tested positive with whole mycobacterial antigen and were negative with peptide, who were then negative (for antigen and peptide) at a subsequent time point. Thus a T cell response to antigen only  
25 predicts that the individual will clear infection. This shows that it is advantageous to combine the first aspect of the invention (testing with protein and peptide) and the second aspect of the invention (testing at two time points) to identify individuals who are recently exposed but have cleared infection.

Thus in one embodiment the invention provides a method of diagnosing an  
30 individual who has been recently exposed to a mycobacterium and is likely to have cleared the infection caused by the mycobacterium, said method comprising (i) determining in vitro whether the T cells of the individual recognise a protein from said

agent having a length of at least 30 amino acids, to a greater extent than one or more peptide epitopes from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent; and (ii) determining whether the T cells of the individual recognise antigen from the mycobacterium at a subsequent  
5 second time point, wherein a greater extent of recognition of the protein in (i) and the finding that the T cells do not recognise antigen at the second time point indicates that the individual has been recently exposed to a mycobacterium and has cleared infection by the mycobacterium.

10 *Methods of diagnosing or monitoring before or during immunosuppressive therapy*

The inventors have realised that detection of latent mycobacterial infection, and therefore of susceptibility to active tuberculosis, in individuals on immunosuppressive therapy may be determined using the T cell based assays described herein.

Accordingly the invention provides a method of diagnosing susceptibility to  
15 active tuberculosis disease and latent mycobacterial infection in an individual on or about to start immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis disease and latent mycobacterial infection.

20 In addition the invention provides a method of monitoring susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis disease and latent  
25 mycobacterial infection.

The individuals who are diagnosed or monitored may be any of the types of individuals who are mentioned herein, but are preferably human.

The immunosuppressive therapy in these methods may comprise administration of an anti-TNF- $\alpha$  agent. Such an agent generally counters the effects of TNF- $\alpha$ . In one  
30 embodiment the anti-TNF- $\alpha$  agent binds to TNF- $\alpha$ . Alternatively the anti-TNF- $\alpha$  agent may act at the TNF- $\alpha$  receptor, and in one embodiment binds the TNF- $\alpha$  receptor. In a preferred embodiment the anti-TNF- $\alpha$  agent is, or comprises, an

antibody or a fragment of an antibody that binds to TNF- $\alpha$ . Such an agent may be a human or mouse antibody or a chimeric antibody which comprises human antibody sequence. In a preferred embodiment the antibody is a human-murine chimeric antibody. In a highly preferred embodiment the antibody is infliximab (remicade) or humira. The anti-TNF- $\alpha$  agent may be, or comprise, a TNF- $\alpha$  receptor or fragment of such a receptor, for example etanercept.

The immunosuppressive therapy may comprise administration of anti-methotrexate, azathioprine, a corticosteroid or mycophenolate mofetil immunosuppressive therapy. The individuals may additionally be taking clacineurin inhibitors, such as cyclosporine or tacrolimus. The individuals may be taking functional analogues of methotrexate, azathioprine, a corticosteroid, mycophenolate mofetil, cyclosporine or tacrolimus which are able to cause immunosuppression.

The said determining of T cell recognition may be carried out using any suitable method, such as any suitable method disclosed herein. The mycobacterium may be any such mycobacterium mentioned herein, but is preferably *M. tuberculosis*. The antigen may be any antigen which is recognised by T cells (such as any type of T cell mentioned herein) and thus may be any of the proteins or peptide epitopes mentioned herein. Analogues of the antigen may be used in the determination such as any of the types of, or specific, analogues mentioned herein.

A kit for carrying out the above methods of diagnosing/monitoring susceptibility to mycobacterial disease/infection is also provided, as well as method of treating individuals who are identified as being susceptible to mycobacterial disease/infection.

#### *Method of detecting susceptibility to onset of mycobacterial disease*

The invention provides a method of detecting susceptibility to onset of active mycobacterial disease in an individual who does not have any symptoms of mycobacterial disease comprising determining whether the individual has increased levels of T cells which recognise a mycobacterial antigen, to thereby determine whether the individual is susceptible to onset of active mycobacterial disease.

The individuals who are tested may be any of the types of individuals who are mentioned herein, but are preferably human. The determining of T cell level may be

carried out using any suitable method, such as any suitable method disclosed herein. The mycobacterium may be any such mycobacterium mentioned herein, but is preferably *M. tuberculosis*. The antigen may be any mycobacterial antigen which is recognised by T cells (such as any type of T cell mentioned herein) and thus may be  
5 any of the proteins or peptide epitopes mentioned herein. Analogues of the antigen may be used in the determination such as any of the types of, or specific, analogues mentioned herein.

The individual may be selected as being susceptible to the onset of disease if the level (frequency) of mycobacterial antigen specific T cells in the individual is at least  
10 five-fold higher, such as at least eight-fold or at least ten-fold higher than at a previous time point in the same individual (generally a previous time point when the individual was also latently infected and asymptomatic). Thus the increase in level of such T cells may be an increase of about at least 80 per million peripheral blood mononuclear cells, such as at least 100 per million, 150 per million or 200 per million.

15 This one embodiment T cells levels are determined at least two points. The first time point which is previous to the above discussed determination of the increase in level of T cells (which herein is defined as determination at the second time may be separated from the second time point by about 3 to 104 weeks, such as about 8 to 52 or 15 to 30 weeks.

20 Individuals identified by the method as susceptible to onset of disease may be given appropriate therapy, such as administration of an anti-mycobacterial agent. Individuals identified by the method as susceptible to onset of disease may be selected for testing with other diagnostic tests for active mycobacterial disease, such radiological investigations (and then may be given appropriate therapy for the active  
25 disease).

A kit for carrying out the method is also provided, as well as method of treating individuals who are identified as being susceptible to the onset of mycobacterial disease.

30 Sequence of ESAT-6:

MTEQQWNFAGIEAAASAIQGNVTSIHSLLEDEGKQSLTKL  
AAAWGGSGSEAYQGVQQKWDATATELNNALQNLARTI

SEAGQAMASTE GNV TGMFA

Sequence of CFP-10:

MAEMKTDAAATLAQEAGNFERISGDLKTQIDQVESTAGSL  
 5 QGQWRGAAGTAAQA AVRFQEAANKQKQELDEISTNIR  
 QAGVQYSRADEEQQALSSQM GF

The invention is illustrated by the following Examples:

#### Example 1

#### 10 Methods

##### **Ex vivo ELISPOT assays**

ELISPOT assays were performed 2-4 hr after venepuncture. Samples were processed and scored by two scientists without reference to personal identifiers or TST results. Peripheral blood mononuclear cells (PBMC) were separated from heparinized  
 15 blood by standard density centrifugation and washed in RPMI. PBMC were counted in an automated cell counter under a microscope, resuspended in complete medium (R10), and plated at  $2.5 \times 10^5$  cells per well in ELISPOT plates pre-coated with catcher anti-IFN- $\gamma$  monoclonal antibody (mAb) (Mabtech, Stockholm, Sweden) and pre-blocked with R10.

20 Duplicate wells contained no antigen (negative control), phytohaemagglutinin (positive control) (ICN Biomedicals, OH, USA), recombinant ESAT-6 (rESAT-6) or one of 12 different peptide pools derived from ESAT-6 and CFP10. Assays were incubated overnight at 37C, 5% CO<sub>2</sub>, and developed the next morning by washing the plates with phosphate buffered saline 0.05% Tween-20 (Sigma, MO, USA), incubating  
 25 for 90 min with detector anti-IFN- $\gamma$  mAb preconjugated to alkaline phosphatase (Mabtech), repeat washing and 15 min incubation with BCIP/NBT<sup>PLUS</sup> chromogenic substrate (Moss Inc, MD, USA). Plates were air dried after washing in tap water.

Assays were scored in an automated ELISPOT counter with the same settings for all samples. Test wells were scored as positive if they contained a mean of at least 5  
 30 spot forming cells (SFCs) more than the mean of the negative control wells, and, in addition, this number was at least twice the mean of the negative control wells. For peptide pools, a positive was defined as response to pools in both arrays as each array

contained a full set of peptides. A positive response to pools of ESAT-6-derived peptides, pools of CFP10-derived peptides or rESAT-6 was deemed a positive ELISPOT assay.

## 5 Peptides

As previously described, 17 peptides spanning the length of the ESAT-6 molecule and 18 peptides spanning the length of the CFP10 molecule were purchased (Research Genetics, AL, USA). Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues; purity was >70%. Peptides were arranged into 12  
10 pools comprising 2 arrays of 6 pools each where each array contained all 35 peptides from the two molecules in contrasting combinations, so that each peptide was tested in quadruplicate.

## Results

15 124 individuals from an Italian hospital with recent (11 weeks previously) exposure to (and therefore risk of infection with) M. tuberculosis were tested using the ELISPOT assay described above. Using the pools of peptides and the whole antigens for both ESAT-6 and CFP-10 only 10 (8%) were found to be positive by tuberculin skin test whereas 34 (27%) were found to be positive using ELISPOT showing that  
20 ELISPOT detects infection earlier after exposure. Of the 124 individuals, 35 were health care workers (HCWs), several of whom may have been previously exposed to M. tuberculosis in the distant past, and 89 were mothers and their 11 week old babies, of whom no babies and only very few mothers might have been previously exposed. Of the 18 HCWs who responded to the ELISPOT 11 (61%) responded to  
25 whole antigen only. Of the 16 mothers and babies who responded 14 (87.5%) responded responded to whole antigen to a greater extent than than to the pools of peptides. In this case the 16 mothers and babies responded only to whole antigen and did not respond to the pools of peptides.

In contrast in a study of 545 children in a M. tuberculosis outbreak at a UK  
30 secondary school, where children were exposed to M. tuberculosis 4 to 12 months prior to testing by ELISPOT, 133 children responded to whole ESAT-6 or peptides from ESAT-6, and from these only 13 (9.8%) responded to whole ESAT-6 only (compared



with the above figures of 61% and 87.5% in the Italian study). This reflects the fact that the children in the UK school were exposed much earlier than the individuals in the Italian study. Therefore the detection of a higher response to whole antigen than to peptides may be used to detect recent exposure to a pathogen.

5

### Example 2

#### Results of two stage testing (two test ELISPOT format) at 3 and 6 months after M. tuberculosis exposure

A T cell recognition based assay (ex vivo ELISPOT) was used to study the  
10 dynamics of early infection at the T cell level (in contrast to symptom based infection/disease indicators). The detectable presence in an individual of effector T cells is believed to indicate that the host is currently infected by a pathogen.

The donors were 108 mothers and babies from the Italian hospital study described above. It was found that 61 donors who had tested negative in the ELISPOT  
15 assay at 3 months tested negative again at 6 months. In addition 15 donors who had tested positive at 3 months also tested positive at 6 months. However 13 donors who had tested negative at 3 months tested positive at 6 months, and 19 donors who had tested positive at 3 months then tested negative at 6 months. Therefore by the use of assaying T cells of individuals at two time points after exposure two important groups  
20 of individuals were identified.

Firstly the 19 donors who tested positive at the first time point and negative at the second time point are individuals initially became infected and then cleared the infection. These individuals will not require treatment. This illustrates the importance of testing a subsequent second time after an initial positive testing when using T cell  
25 based diagnosis of infection. Interestingly all 19 of these donors had only tested positive with antigen and not with peptides, implying that if an individual tests positive in an ELISPOT assay using protein antigen and at the same time negative in an ELISPOT using peptides then the individual may be more likely to clear infection. This illustrates the importance of identifying such individuals (and probably testing  
30 them at subsequent time points) to determine whether or not they will be likely to require treatment.

Secondly the 13 donors who tested negative at the first time point and positive

at the second time point are individuals who have mounted a slower (weaker) T cell response. Such individuals with weaker responses are more likely to fail to control infection, and hence to develop active disease. Thus a T cell based two time point diagnostic test can be used to identify individuals who are more likely to progress to active disease. Clearly it is desirable to target these individuals for therapy. This finding may also be used to identify individuals who mount a slower response to vaccines or to other moieties which induce a cellular response.

### Example 3

#### 10 Early diagnosis of subclinical mycobacterial infection in an immunosuppressed individual

The *ex vivo* enzyme-linked immunospot assay for interferon-gamma (ELISPOT) detects T cells that are specific for antigens expressed by *M. tuberculosis*, but absent from *M. bovis* BCG. In recent tuberculosis (TB) contacts, the assay  
15 correlates significantly more closely with *M. tuberculosis* exposure than the TST, and, unlike the TST, is independent of BCG vaccination status. Thus, it appears to have a higher sensitivity and specificity than the TST for detecting *M. tuberculosis* infection. This is the first clinical application of this assay to a difficult and common clinical problem: the evaluation of a recent TB contact on immunosuppressive therapy.

20 A 24 year old female illegal immigrant from Moldova delivered a healthy baby at the University Hospital of Modena, Modena, Italy. Although she was noted to be thin and persistently coughing, chest radiography was delayed until one week after delivery. X-ray and high resolution computed tomography (HRCT) of the lungs were highly suggestive of active pulmonary TB, and when informed of her suspected diagnosis, she  
25 provided a full medical history. It now transpired that her fever and cough had been present for four months, but anxiety about her status as an illegal immigrant had prevented her from seeking medical attention earlier. Ten years previously in Moldova, she had been treated for pulmonary TB with 2 unspecified oral drugs for about 2 months. Three sputum samples were strongly positive (3+) for acid fast bacilli on  
30 Ziehl-Neelsen (ZN) staining and HIV serology was negative. Standard 4-drug anti-TB therapy was started. Three weeks later, the sputum specimens grew *M. tuberculosis* complex resistant to isoniazid and rifampin. Therapy was therefore switched to a 5-

drug regimen (pyrazinamide, moxifloxacin, ethambutol, streptomycin and clofazimine), which resulted in progressive clinical improvement. The duration of her symptoms suggested that she had been infectious for four months; the investigation of her close contacts was therefore a matter of priority.

5           The most highly exposed contact was her 41-year old husband. He was on long-term immunosuppressive therapy for inactive Crohn's disease with a maintenance dose of azathioprine (150 mg/day). He had no symptoms whatsoever and physical examination was normal. In view of his azathioprine therapy, a complete blood count and differential white cell count were performed, and both were normal. As a close  
10 household contact, the husband was considered to be at high risk of infection with multidrug resistant (MDR) *M. tuberculosis*; if infected, he would be at high risk of progression to active MDR TB, on account of his immunosuppressive therapy. However, the limitations of the TST presented some serious obstacles to his management. In particular, the TST is often falsely negative (poor sensitivity) in  
15 individuals on immunosuppressive medications, with HIV infection, or with certain chronic illnesses (e.g. chronic renal failure), i.e. precisely those people at greatest risk of progression to active tuberculosis. Early identification of infection with MDR *M. tuberculosis* is especially important, since active, symptomatic MDR TB carries a high mortality. The husband was therefore invited to undergo testing by ELISPOT as well as  
20 TST.

TST was administered by the Mantoux method using 5 IU of protein purified derivative (PPD) (Biocine, Chiron Italy). The transverse diameter of cutaneous induration was measured with a ruler and recorded 72 hours after inoculation, using 5  
25 mm as the cut-off for a positive test. Immediately after TST administration, a venous blood sample was taken and the ELISPOT assay performed as previously described, using antigens highly specific for *M. tuberculosis* complex. The antigens used were recombinant early secretory antigenic target-6 (ESAT-6), recombinant culture filtrate protein 10 (CFP10), and peptide pools derived from these antigens.

TST induration was 4 mm, and hence deemed to be negative, whereas the  
30 ELISPOT test result was positive. On account of the positive ELISPOT result, the husband underwent chest radiography and HRCT. Chest radiography showed poorly defined non-specific shadowing in the periphery of the upper zone of the right lung and

chest HRCT demonstrated several small foci of consolidation, one with very early cavitation. Fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) was performed in the anterior segment of the right upper lobe. Lavage fluid revealed acid-fast bacilli on ZN staining, and the patient was therefore prescribed the same 5 anti-TB drugs as his wife, on the basis of a presumptive diagnosis of MDR-TB. *M. tuberculosis* complex was isolated from BAL fluid cultures 5 weeks later. The drug resistance pattern was the same as the wife's isolate, and molecular strain typing (DNA fingerprinting), using IS6110 restriction fragment length polymorphism analysis, indicated that his isolate was identical to that of his wife.

10 Clinical application of this novel T cell-based test in the evaluation of a recent TB contact resulted in the early diagnosis and prompt treatment of sub-clinical, active pulmonary MDR TB in an asymptomatic person with a negative skin test. As well as being of direct benefit to the husband, early diagnosis prevented secondary transmission of this MDR *M. tuberculosis* strain in the community. The reason why the  
15 ELISPOT assay was able to detect the presence of early subclinical MDR TB where the TST failed to do so may be because ELISPOT assay may be less susceptible than the TST to false negative results in iatrogenically immunosuppressed individuals. A large and increasing number of patients are on medications that cause mild-to-moderate immunosuppression and, as in the case reported here, many have impaired delayed type  
20 hypersensitivity responses and falsely negative TSTs. Moreover, it is often precisely these immunosuppressed patients who are more likely to progress to severe and disseminated forms of TB. Screening for asymptomatic *M. tuberculosis* infection is especially critical in patients with autoimmune and inflammatory diseases who are candidates for therapy with anti-TNF-alpha agents (e.g. Infliximab). An important  
25 adverse effect of this potent new class of agents is reactivation of TB in latently infected individuals, but diagnosing latent *M. tuberculosis* infection by TST in these patients is especially difficult as most are already on immunosuppressive agents.

We have shown that this novel T cell-based test detected early, active MDR TB in the absence of symptoms and in the setting of a negative TST. Our report  
30 demonstrates, for the first time, the clinical utility of a blood test for *M. tuberculosis* infection, and it shows the potential of ELISPOT for improving clinical outcome. On the basis of these results, ELISPOT is currently being used to screen all the hospital

contacts of the source case described in this report in order to help prevent a nosocomial outbreak of MDR TB.

#### Example 4

5 Investigation of patients with rheumatoid arthritis on immunosuppressive medications about to start Infliximab therapy

The Th1 cytokine Tumour Necrosis Factor alpha (TNF- $\alpha$ ) is a key cytokine in immunity and inflammation. Monoclonal antibodies that neutralise TNF and drugs that block its receptor are an important new class of drugs for the treatment of chronic  
10 autoimmune diseases that are refractory to other medications. Such conditions include rheumatoid arthritis (RA) and Crohn's disease (CD), and Ankylosing Spondylitis (AS) and the list of conditions where these drugs are finding clinical application is increasing and now includes seronegative spondyloarthropathies and sarcoidosis. The first anti-TNF agent to enter clinical practice was Infliximab, a humanised anti-TNF monoclonal  
15 antibody. Thus it is with this agent that we have the most experience of clinical efficacy, as well as adverse effects.

Infliximab is safe and well tolerated and the main adverse event is the reactivation of latent tuberculosis (TB) infection (LTBI). 90% of people with LTBI remain healthy life-long; only 10% will develop active TB. However, Infliximab  
20 therapy increases this risk many fold, and most patients with LTBI who start Infliximab will develop active TB within 17 weeks of initiating therapy. Reactivation TB can be prevented by taking anti-TB preventative therapy (isoniazid preventative therapy) for 6 months. Doctors prescribing Infliximab are therefore advised by the manufacturer to screen patients for LTBI prior to starting therapy.

25 The difficulty lies in identifying who actually has LTBI. Until very recently, the only method for diagnosing LTBI was the century-old tuberculin skin test (TST). The TST has some major drawbacks, including low specificity (mainly due to cross-reactivity with *M. bovis* BCG and environmental mycobacteria) and low sensitivity (in fact, there is no gold standard for the diagnosis of LTBI). The main problems faced by  
30 physicians who wish to start Infliximab therapy are thus:

- False negative TST results (caused by the existing immunosuppressive therapy)

or indeed the target disease itself). False negative results mean that patients with true LTBI will be missed, will start Infliximab, and then develop reactivation TB

- False positive TST results (caused by prior BCG-vaccination). False positive results mean that many patients in need of Infliximab will be wrongly denied therapy for fear of reactivating TB, although in reality they do not have LTBI. This is a common problem, as most of the population of Europe and the world is BCG-vaccinated

The ELISPOT assay was used to study 10 rheumatoid arthritis patients about to start Infliximab therapy. Despite already being on immunosuppressive therapies (including methotrexate, azathioprine, corticosteroids) these patients still had readily detectable IFN- $\gamma$ -secreting T cells to positive control antigens in the ELISPOT assay. One of these patients had a past history of household TB exposure. She was positive for TB infection by ELISPOT, but negative by TST. This person's LTBI would therefore have been missed if only TST had been used, but it was detected by ELISPOT. Moreover, 10 weeks after starting Infliximab she remained ELISPOT positive, indicating that ELISPOT can be used to detect LTBI even after a patient has started TNF-blockade therapy.

#### Example 5

#### Investigation of effector T cell frequency as predictor of onset of tuberculosis disease

The ELISPOT assay was used to examine the change in effector T cell frequency in a patient who progressed from asymptomatic latent tuberculosis infection to active tuberculosis. Onset of symptoms occurred at 8 months after the start of the study, and treatment of disease was started at 10 months. The diagnosis of disease was confirmed by culturing of the mycobacterium. The results are shown below.

Time point (months)	Effector T cell frequency (SFU's per $10^6$ cells)
0	40
6	564
12	216

SFU- spot forming units

The above data shows that there is a marked increase in effector T cell  
5 frequency (at 6 months) before the onset of disease symptoms (at 8 months). The  
increase in effector T cell frequency probably reflects an increase in the burden of  
bacilli (i.e. an increase in antigen burden). Although the increase in bacilli leads to an  
increase in effector T cells it does not cause enough tissue pathology to cause  
symptoms until the 8 month time point. Therefore detection of effector T cells (for  
10 example using ELISPOT) may be used as a predictor of the onset of active  
tuberculosis.

CLAIMS

1. Method of diagnosing in an individual recent exposure to an agent which is a pathogen, vaccine or any other moiety which induces a cellular response, said method comprising determining in vitro whether the T cells of the individual recognise a  
5 protein from said agent having a length of at least 30 amino acids, to a greater extent than one or more peptide epitopes from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.
2. Method according to claim 1 comprising determining whether T cells of the  
10 individual exhibit a greater reaction to a protein from said agent having a length of at least 30 amino acids than to one or more peptide epitopes from the agent, a greater reaction indicating that the individual has recently been exposed to the agent.
3. Method according to claim 1 wherein determining whether the T cells recognise said  
15 protein is performed by determining the reaction of the T cells to an analogue of the protein which is recognised by T cells which recognise said protein, wherein said analogue has a length of at least 30 amino acids.
4. Method according to claim 1 or 3 wherein determining whether the T cells recognise  
20 said peptide epitope is performed by determining the reaction of the T cells to an analogue of the peptide epitope which analogue is recognised by T cells which recognise said peptide epitope.
5. Method according to any one of the preceding claims comprising:  
25 (i) contacting a first population of T cells from the individual with (a) one or more peptide epitopes from the agent, or (b) an analogue of said peptide(s) which is recognised by T cells that recognise said peptide(s), and determining the reaction of the T cells to the peptide(s) or analogue(s), and  
(ii) contacting a second population of T cells from the individual with (a) a protein  
30 from the agent, or (b) an analogue of said protein which is recognised by T cells that recognise said protein, wherein the protein or analogue has a length of at least 30 amino acids and determining the reaction of the T cells to the protein or analogue.



6. Method according to any one of the preceding claims in which the individual is diagnosed as having been exposed to the agent recently if there is substantially no reaction of the T cells to the peptide epitope or an analogue thereof.

5

7. Method according to any one of the preceding claims in which protein or its analogue comprises at least the amino acid sequence of the peptide epitope or its analogue.

10 8. Method according to any one of the preceding claims in which the peptide epitope, or the analogue of the peptide epitope, has a length of 8 to 29 amino acids.

9. Method according to any one of the preceding claims wherein whether or not the T cells recognise a pool of at least 4 peptide epitopes, or analogues thereof, is determined.

15

10. Method according to any one of the preceding claims wherein a pool of peptide epitopes and/or analogues which together represent all of the possible epitopes from the protein is used.

20 11. Method according to any one of the preceding claims in which during detection of the reaction of the T cells to the protein, or the analogue of the protein, antigen presenting cells are present which are capable of processing the protein and presenting it to the T cells.

25 12. Method according to any one of the preceding claims wherein the pathogen is an intracellular pathogen or the vaccine is against an intracellular pathogen.

13. Method according to any one of the preceding claims wherein the pathogen is HPV, HIV, SIV, HCV, a Chlamydia species, HBV, EBV, CMV, VZV, HSV,

30 Legionella, S. typhi, P. falciparum, Leishmaniasis, M. leprae, influenza virus, foot and mouth virus, a Toxoplasma species, a Brucella species, a Cryptococcus species, a Candida species or an Aspergillus species; or the vaccine is against any of these

pathogens.

14. Method according to any one of claims 1 to 12 wherein the pathogen is M. tuberculosis or the vaccine is against M. tuberculosis.

5

15. Method according to any one of the preceding claims wherein the protein and/or epitope peptide is from ESAT-6 or CFP10.

16. Method according to any one of the preceding claims wherein the peptide(s) is chosen from one or more of the following peptide epitopes:

10 MTEQQWNFAGIEAAA  
WNFAGIEAASAIQG  
IEAASAIQGNVTSI  
SAIQGNVTSIHSLLD  
15 NVTSIHSLLDGKQS  
HSLLDGKQSLTKLA  
EGKQSLTKLAAAWGG  
LTKLAAAWGGSGSEA  
AAWGGSGSEAYQGVQ  
20 SGSEAYQGVQQKWD  
YQGVQQKWDATATEL  
QKWDATATELNNALQ  
TATELNNALQNLART  
NNALQNLARTISEAG  
25 NLARTISEAGQAMAS  
ISEAGQAMASTEGNV  
QAMASTEGNV TGMFA  
MAEMKTD AATLAQEA  
TDAATLAQEAGNFER  
30 LAQEAGNFERISGDL  
GNFERISGDLKTQID  
ISGDLKTQIDQVEST

KTQIDQVESTAGSLQ  
QVESTAGSLQGQWRG  
AGSLQGQWRGAAGTA  
GQWRGAAGTAAQAAV  
5 AAGTAAQAAVVRFQE  
AQAAVVRFQEAANKQ  
VRFQEAANKQKQELD  
AANKQKQELDEISTN  
KQELDEISTNIRQAG  
10 EISTNIRQAGVQYSR  
IRQAGVQYSRADEEQ  
VQYSRADEEQQQALS  
ADEEQQQALSSQMGF

or an analogue thereof which is recognised by a T cell which recognises the peptide  
15 epitope.

17. Method according to any one of the preceding claims wherein recognition of the peptide epitope, or its analogue, or of the protein, or its analogue, is determined by detecting secretion of a cytokine from the T cells.

20

18. Method according to claim 17 in which the cytokine is IFN- $\gamma$ .

19. Method according to claim 17 or 18 in which the cytokine is detected by allowing the cytokine to bind to an immobilised antibody specific to the cytokine and then  
25 detecting the presence of the antibody/cytokine complex.

20. Method of diagnosing in an individual recent exposure to an agent which is pathogen, vaccine or any other moiety which induces a cellular response, said method comprising determining in vivo whether the T cells of the individual recognise a  
30 protein from said agent having a length of at least 30 amino acids, to a greater extent than a peptide epitope from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

21. Method according to claim 20 which is performed using the method of any one of claims 2 to 19.

5 22. Use a protein having a length of at least 30 amino acids from an agent which is a pathogen, vaccine or any other moiety which induces a cellular response and/or one or more peptide epitopes from the agent in the manufacture of a diagnostic means for use in a method of diagnosing in an individual recent exposure to the agent, said method comprising determining whether the T cells of the individual recognise the protein to a  
10 greater extent than peptide epitope(s), a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

23. A product comprising a protein from an agent which is a pathogen, vaccine or any other moiety which induces a cellular response, said protein having a length of at least  
15 30 amino acids, and/or one or more peptide epitopes from the agent for separate, simultaneous or sequential use in a method of diagnosing in an individual recent exposure to the agent, said method comprising determining whether the T cells of the individual recognise the protein to a greater extent than the peptide epitope(s), a greater extent of recognition of the protein indicating that the individual has recently been  
20 exposed to the agent.

24. Method of treating an individual comprising administering to an individual diagnosed as having been exposed recently to a pathogen by a method according to any of the preceding claims, a product which prevent or treats the condition caused by the  
25 pathogen.

25. Use of a product which prevents or treats a condition caused by a pathogen in the manufacture of a medicament for the therapy of an individual who has been diagnosed as having been recently exposed to the pathogen by a method according to any one of  
30 claims 1 to 21.

26. Method or use according to claim 24 or 25 wherein the pathogen is M. tuberculosis

and/or the agent is rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicylic acid, kanamycin, capreomycin, ethionamide, cycloserine, thiacetazone or a fluoroquinolone, or an analogue of such an agent.

- 5 27. A kit for carrying out the method of any one of claims 1 to 21 comprising (i) said epitope peptide or said analogue thereof, and (ii) said protein or said analogue thereof, and optionally also a means to detect whether T cells recognise (i) and (ii).
28. A kit according to claim 27 which also comprises an agent as defined in any one of  
10 claims 24 to 26.
29. Method of diagnosing an individual who has cleared an infection by a pathogen comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the  
15 pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the individual has cleared the infection.
30. Method of diagnosing an individual who is more likely to progress to active disease after exposure to a pathogen comprising determining whether the T cells of the  
20 individual recognise antigen from the pathogen at a first and subsequent second time point after exposure to the pathogen, wherein the finding that the T cells do not recognise the antigen at the first time point, but do recognise the antigen at the second time point indicates that the individual is more likely to progress to active disease.
- 25 31. Method of diagnosing an individual who mounts a weaker response to a vaccine or a moiety which induces a cellular response after exposure to the vaccine or moiety comprising determining whether the T cells of the individual recognise antigen from the vaccine or moiety at a first and subsequent second time point after exposure, wherein the finding that the T cells do not recognise the antigen at the first time point,  
30 but do recognise the antigen at the second time point indicates that the individual is mounting a weaker response to the vaccine or moiety.

32. Method of testing the efficacy of a vaccine which has been administered to an individual comprising determining whether the T cells of the individual recognise antigen from the pathogen at a first and a subsequent second time point after exposure to the pathogen, wherein the finding that the T cells recognise antigen at the first time point and not at the second time point indicates that the vaccine antigen has been  
5 cleared and is not persisting.

33. Method according to any one of claims 29 to 32 wherein the first time point and second time point are separated by about 12 weeks.

10

34. Method according to any one of claims 29 to 33 wherein the first time point is about 2 to 16 weeks after exposure and/or the second time point is about 18 to 48 weeks after exposure.

15 35. Method according to any one of claims 29 to 34 wherein said determining is performed by detecting whether the T cells of the individual recognise/react to an antigen from said pathogen, vaccine or moiety.

20 36. Method according to any one of claims 29 to 35 wherein the antigen is a protein from said pathogen, vaccine or moiety having a length of at least 30 amino acids or is a peptide epitope from the pathogen, vaccine or moiety, wherein the protein or peptide epitope are as defined in any one of the preceding claims.

25 37. Method according to any one of claims 29 to 36 wherein said determining is performed by detecting the reaction of the T cells to an analogue of antigen which is optionally an analogue as defined in any one of the preceding claims.

30 38. Method according to any one of claims 29 to 37 wherein said determining is performed by contacting in vitro or in vivo a population of T cells from the individual with the antigen, or an analogue of said antigen and determining the reaction of the T cells to the antigen or analogue.

39. Method according to any one of claims 29 to 38 wherein the determination is deemed negative if there is substantially no reaction of the T cells to the antigen or analogue.

5 40. Method according to any one of the claims 29 to 39 wherein said determining comprises detecting whether or not the T cells recognise a pool of a least 4 peptide epitopes, or analogues thereof.

41. Method according to any one of claims 29 to 40 wherein during said determining  
10 antigen presenting cells are present which are capable of processing the antigen or analogue and presenting it to the T cells.

42. Method according to any one of claims 29 to 41 wherein the pathogen is an intracellular pathogen or the vaccine is against an intracellular pathogen.

15

43. Method according to any one of claims 29 to 42 wherein the pathogen is HPV, HIV, SIV, HCV, a Chlamydia species, HBV, EBV, CMV, VZV, HSV, Legionella, S. typhi, P. falciparum, Leishmaniasis, M. leprae, influenza virus, foot and mouth virus, a Toxoplasma species, a Brucella species, a Cryptococcus species, a  
20 Candida species or an Aspergillus species; or the vaccine is against any of these pathogens.

44. Method according to any one of claims 29 to 42 wherein the pathogen is M. tuberculosis or the vaccine is against M. tuberculosis.

25

45. Method according to any one of claims 29 to 44 wherein recognition of the antigen or analogue is determined by detecting secretion of a cytokine from the T cells.

46. Method according to claim 45 in which the cytokine is IFN- $\gamma$ .

30

47. Method according to claim 45 or 46 in which the cytokine is detected by allowing the cytokine to bind to an immobilised antibody specific to the cytokine and then

detecting the presence of the antibody/cytokine complex.

48. Method of treating an individual comprising administering to an individual diagnosed as being infected by a pathogen by a method according to any of claims 30 to 47, a product which prevent or treats the condition caused by the pathogen.

49. Method according to claim 48 wherein the pathogen is *M. tuberculosis* and/or the agent is rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-aminosalicylic acid, kanamycin, capreomycin, ethionamide, cycloserine, thiacetazone or a fluoroquinolone, or an analogue of such an agent.

50. Use of an antigen or analogue as defined in any one of claims 29 to 47 in the manufacture of a diagnostic means for use in a method according to any one of claims 29 to 47 wherein the method is performed in vivo.

51. A kit for carrying out the method of any one of claims 29 to 47 comprising the antigen or analogue, and optionally also a means to detect whether T cells recognise antigen or analogue.

52. A kit according to claim 51 which also comprises an agent as defined in claim 48 or 49.

53. Method of diagnosing susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on or about to start immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to active tuberculosis disease and latent mycobacterial infection.

54. Method of monitoring susceptibility to active tuberculosis disease and latent mycobacterial infection in an individual on immunosuppressive therapy comprising detecting whether or not the T cells of the individual recognise mycobacterial antigen, wherein recognition of mycobacterial antigen by the T cells indicates susceptibility to



active tuberculosis disease and latent mycobacterial infection.

55. Method according to claim 53 or 54 wherein the immunosuppressive therapy comprises administration of an anti-TNF- $\alpha$  antibody, methotrexate, azathioprine, a  
5 corticosteroid or mycophenolate mofetil .

56. Method according to claim 54 wherein the antibody is infliximab.

57. Method according to any one of claims 53 to 56 wherein T cell recognition is  
10 performed using a method as defined in any one of the preceding claims.

58. Method according to any one of claims 53 to 57 comprising determining in vitro whether the T cells of the individual recognise/react to a mycobacterial antigen or an analogue of the mycobacterial antigen.  
15

59. Method according to any one of claims 53 to 58 comprising contacting a population of T cells from the individual with (a) one or more peptide epitopes of a mycobacterium, or (b) an analogue of said peptide(s) which is recognised by T cells that recognise said peptide(s), and determining the reaction of the T cells to the  
20 peptide(s) or analogue(s).

60. Method according to any one of claims 53 to 59 in which the peptide epitope, or the analogue of the peptide epitope, has a length of 8 to 100 amino acids.

25 61. Method according to any one of claims 53 to 60 wherein whether or not the T cells recognise a pool of a least 4 peptide epitopes, or analogues thereof, is determined.

62. Method according to any one of claims 53 to 61 in which during detection of the reaction of the T cells to the peptide, or the analogue of the peptide, antigen presenting  
30 cells are present which are capable of processing the peptide or analogue and presenting it to the T cells.

63. Method according to any one of claims 53 to 62 wherein the mycobacterial antigen is of *M. tuberculosis*.

64. Method according to any one of claims 53 to 63 wherein the mycobacterial antigen  
5 is ESAT-6 or CFP10.

65. Method according to any one of claims 53 to 64 wherein recognition by the T cells of a peptide or peptides chosen from one or more of the following peptide epitopes is determined:

10

MTEQQWNFAGIEAAA  
X WNFAGIEAAASAIQG  
IEAAASAIQGNVTSI  
SAIQGNVTSIHSLLD  
15 NVTSIHSLLDDEGKQS  
HSLLDDEGKQSLTKLA  
EGKQSLTKLAAAWGG  
LTKLAAAWGGSGSEA  
AAWGGSGSEAYQGVQ  
20 SGSEAYQGVQQKWDA  
YQGVQQKW DATATEL  
QKW DATATELNNALQ  
TATELNNALQNLART  
X NNALQNLARTISEAG  
25 NLARTISEAGQAMAS  
ISEAGQAMASTE GNV  
QAMASTE GNV TGMFA  
MAEMKTDAATLAQEA  
TDAATLAQEAGNFER  
30 LAQEAGNFERISGDL  
X GNFERISGDLKTQID  
ISGDLKTQIDQVEST

KTQIDQVESTAGSLQ  
 QVESTAGSLQGQWRG  
 AGSLQGQWRGAAGTA  
 GQWRGAAGTAAQAAV  
 5 AAGTAAQAAVVRFQE  
 AQAADVRFQEAANKQ  
 VRFQEAANKQKQELD  
 AANKQKQELDEISTN  
 KQELDEISTNIRQAG  
 10 EISTNIRQAGVQYSR  
 IRQAGVQYSRADEEQ  
 VQYSRADEEQQALS  
 ADEEQQALSSQMGF

or an analogue thereof which is recognised by a T cell which recognises the peptide  
 15 epitope.

66. Method according to any one of claims 53 to 65 wherein T cell recognition is determined by detecting secretion of a cytokine from the T cells.

20 67. Method according to claim 66 in which the cytokine is IFN- $\gamma$ .

68. Method according to claim 66 or 67 in which the cytokine is detected by allowing the cytokine to bind to an immobilised antibody specific to the cytokine and then detecting the presence of the antibody/cytokine complex.

25

69. Method of treating an individual comprising administering to an individual diagnosed as being susceptible to active tuberculosis disease or latent mycobacterial infection by a method according to any of claims 53 to 68, a product which prevents or treats said disease or infection.

30

70. Method according to claim 69 wherein the product is rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicylic acid, kanamycin,

capreomycin, ethionamide, cycloserine, thiacetazone or a flouroquinolone, or an analogue of such a product.

71. A kit for carrying out the method of any one of claims 53 to 68 comprising a  
5 mycobacterial antigen or an analogue thereof, and optionally also a means to detect whether T cells recognise the mycobacterial antigen or analogue.

72. A kit according to claim 70 wherein the mycobacterial antigen or analogue thereof comprises a peptide epitope which is 8 to 100 amino acids long.

10

73. A kit according to claim 71 or 72 which also comprises a product as defined in claim 69 or 70.

74. Method of detecting onset of active mycobacterial disease, or susceptibility to  
15 onset of active mycobacterial disease, in an individual who does not have any symptoms of mycobacterial disease comprising determining whether the individual has increased levels of T cells which recognise a mycobacterial antigen, to thereby determine onset of mycobacterial disease or whether the individual is susceptible to onset of active mycobacterial disease.

20

75. Method according to claim 74 comprising determining whether the level of said T cells in the individual is at least five-fold higher compared to a previous time point.

76. Method according to claim 74 or 75 comprising determining whether the level of  
25 said T cells in the individual is at least 100 per million peripheral blood mononuclear cells higher compared to a previous time point.

77. Method according to any one of claims 74 to 76 comprising determining at a first time point the basal level of mycobacterial antigen specific T cells in the individual and  
30 determining at a subsequent second time point whether or not the level of said T cells in the individual has increased.

78. Method according to claim 77 wherein the first and second time point are separated by about 3 to 24 months.

79. Method according to any one of claims 74 to 78 wherein the level of T cells is  
5 detected by measuring in vitro the recognition/reaction of the T cells to a mycobacterial antigen or an analogue of the mycobacterial antigen.

80. Method according to any one of claims 74 to 79 comprising contacting a population of T cells from the individual with (a) one or more peptide epitopes of a  
10 mycobacterium, or (b) an analogue of said peptide(s) which is recognised by T cells that recognise said peptide(s), and determining the reaction of the T cells to the peptide(s) or analogue(s).

81. Method according to any one of claims 74 to 80 in which the peptide epitope, or  
15 the analogue of the peptide epitope, has a length of 8 to 100 amino acids.

82. Method according to any one of claims 74 to 81 wherein the reaction of the T cells to a pool of a least 4 peptide epitopes, or analogues thereof, is determined.

20 83. Method according to any one of claims 74 to 82 in which during detection of the reaction of the T cells to the peptide, or the analogue of the peptide, antigen presenting cells are present which are capable of processing the peptide or analogue and presenting it to the T cells.

25 84. Method according to any one of claims 74 to 83 wherein the mycobacterial antigen is of *M. tuberculosis*.

85. Method according to any one of claims 74 to 84 wherein the mycobacterial antigen is ESAT-6 or CFP10.

30

86. Method according to any one of claims 74 to 85 wherein recognition by the T cells of a peptide or peptides chosen from one or more of the following peptide epitopes is

determined:

MTEQQWNFAGIEAAA  
WNFAGIEAAASAIQG  
5 IEAAASAIQGNVTSI  
SAIQGNVTSIHSLLD  
NVTSIHSLLDDEGKQS  
HSLLDDEGKQSLTKLA  
EGKQSLTKLAAAWGG  
10 LTKLAAAWGGSGSEA  
AAWGGSGSEAYQGVQ  
SGSEAYQGVQQKWD  
YQGVQQKWDATATEL  
QKWDATATELNNALQ  
15 TATELNNALQNLART  
NNALQNLARTISEAG  
NLARTISEAGQAMAS  
ISEAGQAMASTEAGNV  
QAMASTEAGNVGTGMFA  
20 MAEMKTDAAATLAQEA  
TDAATLAQEAGNFER  
LAQEAGNFERISGDL  
GNFERISGDLKTQID  
ISGDLKTQIDQVEST  
25 KTQIDQVESTAGSLQ  
QVESTAGSLQGQWRG  
AGSLQGQWRGAAGTA  
GQWRGAAGTAAQAAV  
AAGTAAQAAVVRFQE  
30 AQAADVRFQEAANKQ  
VRFQEAANKQKQELD  
AANKQKQELDEISTN

KQELDEISTNIRQAG  
EISTNIRQAGVQYSR  
IRQAGVQYSRADEEQ  
VQYSRADEEQQALS  
5 ADEEQQALSSQMGF

or an analogue thereof which is recognised by a T cell which recognises the peptide epitope.

87. Method according to any one of claims 74 to 86 wherein T cell recognition is  
10 determined by detecting secretion of a cytokine from the T cells.

88. Method according to claim 87 in which the cytokine is IFN- $\gamma$ .

89. Method according to claim 87 or 88 in which the cytokine is detected by allowing  
15 the cytokine to bind to an immobilised antibody specific to the cytokine and then detecting the presence of the antibody/cytokine complex.

90. Method of treating an individual comprising administering to an individual diagnosed as being susceptible to active tuberculosis disease or latent mycobacterial  
20 infection by a method according to any of claims 74 to 89, a product which prevents or treats said disease or infection.

91. Method according to claim 90 wherein the product is rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin, para-amino-salicylic acid, kanamycin,  
25 capreomycin, ethionamide, cycloserine, thiacetazone or a fluoroquinolone, or an analogue of such a product.

92. A kit for carrying out the method of any one of claims 74 to 91 comprising a mycobacterial antigen or an analogue thereof, and optionally also a means to detect  
30 whether T cells recognise the mycobacterial antigen or analogue.

93. A kit according to claim 92 wherein the mycobacterial antigen or analogue thereof

comprises a peptide epitope which is 8 to 100 amino acids long.

94. A kit according to claim 92 or 93 which also comprises a product as defined in claim 90 or 91.



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GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
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(54) Title: **DIAGNOSTICS METHOD**

(57) Abstract: Method of diagnosing in an individual recent exposure to an agent which is a pathogen, vaccine or any other moiety which induces a cellular response, said method comprising determining in vitro whether the T cells of the individual recognise a protein from said agent having a length of at least 30 amino acids, to a greater extent than one or more peptide epitopes from the agent, a greater extent of recognition of the protein indicating that the individual has recently been exposed to the agent.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 03/02936

A. CLASSIFICATION OF SUBJECT MATTER  
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According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 26248 A (ISIS INNOVATION ;LALVANI AJIT (GB); PATHAN ANSAR AHMED (GB)) 11 May 2000 (2000-05-11)	1-5, 7-10,12, 14-23, 27,50, 51,53, 57-68, 71,72, 92,93
Y	page 3, line 1-13; claims 1-22; examples 2,3  --- -/-	24-26, 29-47, 54-56, 74-89

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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# INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>PATHAN A A ET AL: "DIRECT EX VIVO ANALYSIS OF ANTIGEN-SPECIFIC IFN-GAMMA-SECRETING CD4 T CELLS IN MYCOBACTERIUM TUBERCULOSIS-INFECTED INDIVIDUALS: ASSOCIATIONS WITH CLINICAL DISEASE STATE AND EFFECT OF TREATMENT" JOURNAL OF IMMUNOLOGY, THE WILLIAMS AND WILKINS CO. BALTIMORE, US, vol. 167, no. 9, 1 November 2001 (2001-11-01), pages 5217-5225, XP001093768 ISSN: 0022-1767 the whole document</p>	<p>24-26, 29-47, 54-56</p>
Y	<p>--- LALVANI A ET AL: "Enhanced contact tracing and spatial tracking of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells" LANCET THE, LANCET LIMITED. LONDON, GB, vol. 357, no. 9273, 23 June 2001 (2001-06-23), pages 2017-2021, XP004247721 ISSN: 0140-6736 abstract</p>	<p>74-89</p>
A	<p>--- WO 01 04151 A (STATENS SERUMINSTITUT ;ANDERSEN PETER (DK); SKJOET RIKKE (DK)) 18 January 2001 (2001-01-18) the whole document</p>	<p>1-94</p>
P,X	<p>--- WO 02 054072 A (ISIS INNOVATION ;LALVANI AJIT (GB)) 11 July 2002 (2002-07-11)  the whole document</p>	<p>1-5, 7-10, 12-20, 22-27, 29-40, 42-49, 51-54, 57-72, 74,77, 79-82, 84-93</p>

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

In International application No.  
PCT/GB 03/02936

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 20-21, 24, 26, 29-50, 53-57, 59-70, 74-78 and 80-91 include diagnostic/therapy methods involving the treatment of the human/animal body covered by the provisions of Rule 67.1 (iv) PCT.
2. ☒ Claims Nos.: 22-25, 27-28, 50-51, 71-73, 92-94  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

Continuation of Box I.2

Claims Nos.: 22-25, 27-28, 50-51, 71-73, 92-94

Present claims 22-25, 27-28, 50-51, 71-73 and 92-94 relate to an extremely large number of possible products which may be obtained from apparently any pathogen. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the products claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to peptide/antigen products obtained from *Mycobacterium tuberculosis*.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-94

diagnostic methods and kits

1.1. Claims: 1-28

methods of diagnosis/therapy in which T-cells recognize proteins derived from a pathogen and having at least 30 amino acids to a greater extent than one or more peptide epitope from the pathogen agent, and products and kits thereof.

1.2. Claims: 29-52

methods of diagnosis/therapy in which T-cells recognize antigens derived from a pathogen at a first and a second time point after exposure to the pathogen, and products and kits thereof.

1.3. Claims: 53-94

methods of diagnosis/therapy in which an increased level of T-cells recognize mycobacterial antigens, and products and kits thereof.

The present application lacks unity (Rule 13.1 PCT) because the above mentioned groups of inventions do not appear to be linked by a common single novel and inventive concept within the sense of Rule 13.2 PCT.

Please note that all inventions mentioned under item 1, although not necessarily linked by a common inventive concept, could be searched without effort justifying an additional fee.

## INTERNATIONAL SEARCH REPORT

 Intern  
 Application No  
 PCT/GB 03/02936

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